Conditional mutation of *Brca1* in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation

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Cre-mediated excision of exon 11 of the breast-tumour suppressor gene *Brca1* in mouse mammary epithelial cells causes increased apoptosis and abnormal ductal development. Mammary tumour formation occurs after long latency and is associated with genetic instability characterized by aneuploidy, chromosomal rearrangements or alteration of *Trp53* (encoding p53) transcription. To directly test the role of p53 in Brca1-associated tumorigenesis, we introduced a *Trp53*-null allele into mice with mammary epithelium-specific inactivation of *Brca1*. The loss of p53 accelerated the formation of mammary tumours in these females. Our results demonstrate that disruption of Brca1 causes genetic instability and triggers further alterations, including the inactivation of p53, that lead to tumour formation.

Introduction

Breast cancer is the most common cancer and the second-leading cause of cancer mortality in women, with approximately one in nine being affected in their lifetime[1,2]. *BRCA1*, located on human chromosome 17q21, was isolated as the gene responsible for increased susceptibility to familial breast and ovarian cancer[3]. Germline mutations of *BRCA1* have been detected in approximately 90% of familial breast and ovarian cancers and approximately 50% of familial breast cancer alone[2,4]. Full-length BRCA1 is a nuclear protein of 220 kD and 1,863 amino acids[3,5]. *BRCA1* also encodes several isoforms of varying sizes which are primarily due to alternative splicing of exons 1 and 11 (refs 6–8). Exon 11 is the largest exon and encodes approximately 60% of the protein. It also contains two putative nuclear-localization signals and interacts indirectly with RAD 51, a homologue of RecA that is involved in DNA-damage repair[3,9–14]. BRCA1 also co-immunoprecipitates with p53 both *in vitro* and *in vivo*, and regulates p53-dependent gene expression[15,16], suggesting a possible interaction between these two tumour-suppressor proteins during tumorigenesis.

In the mouse, *Brca1* encodes a protein of 1,812 amino acids with approximately 58% identity to the human protein[17,18]. The gene is highly expressed in rapidly proliferating mammary epithelial cells during pregnancy and downregulated during lactation[17]. Due to the early embryonic lethality associated with the loss of Brca1 (refs 19–23), however, its role in mammary gland development and neoplasia in mice remains unknown.

To overcome the early lethality and to study the function of Brca1 during mammary gland development and neoplasia, we deleted exon 11 of *Brca1* using a Cre-loxP approach[24]. Mice were engineered to that carry a *Brca1*-null allele, a *Brca1* conditional allele and a *Cre* transgene under the control of the promoter of the gene encoding whey acidic protein (*Wap*) or mouse mammary tumour virus-long terminal repeat (MMTV-LTR), which are active in mammary epithelial cells. Mammary-gland tumours formed after long latency at the expense of genetic stability and alteration of *Trp53* transcription. Notably, removal of one *Trp53* allele accelerated mammary-gland tumour formation. Our study demonstrates that Brca1 functions as a tumour suppressor in the mouse and interacts with p53 during tumorigenesis. It also provides a valuable mouse model to screen for factors that promote or prevent mammary-gland tumour formation.

Conditional mutation of *Brca1* in mouse mammary tissue

The conditional mutation of *Brca1* was achieved by flanking exon 11 of the gene with *loxP* sites (Fig. 1*a*). The presence of the *neo* gene in intron 10 completely blocked normal splicing of *Brca1* and caused embryonic lethality at approximately embryonic day 8.5 (X.X. and C.-X.D., unpublished data). We therefore removed pLoxpneo by transient expression of the Cre recombinase in heterozygous ES cells. Of 384 clones screened for recombination, 2 had the *neo* gene deleted, leaving one *loxP* site in intron 10 and another in intron 11 (Fig. 1*b*). Both clones were injected into blastocysts and germline transmission of the conditional *Brca1* allele was obtained.

We next generated mice carrying a *Brca1*-null allele ($Brca1^{Ko22}$), a conditional allele ($Brca1^{Co}$) or either an *MMTV-Cre* or *Wap-Cre* transgene (Fig. 2*a*). The *MMTV-Cre* transgene is active in many tissues, whereas the *Wap-Cre* transgene is expressed almost exclusively in mammary epithelial cells[25]. We evaluated the extent of *Wap-Cre*–mediated excision of exon 11 and its tissue distribution by PCR and Southern-blot analyses. PCR on multiple tissues and organs demonstrated that the excision of exon 11 occurred almost exclusively in mammary tissue (Fig. 2*b*). Southern-blot analysis of mammary tissue at several

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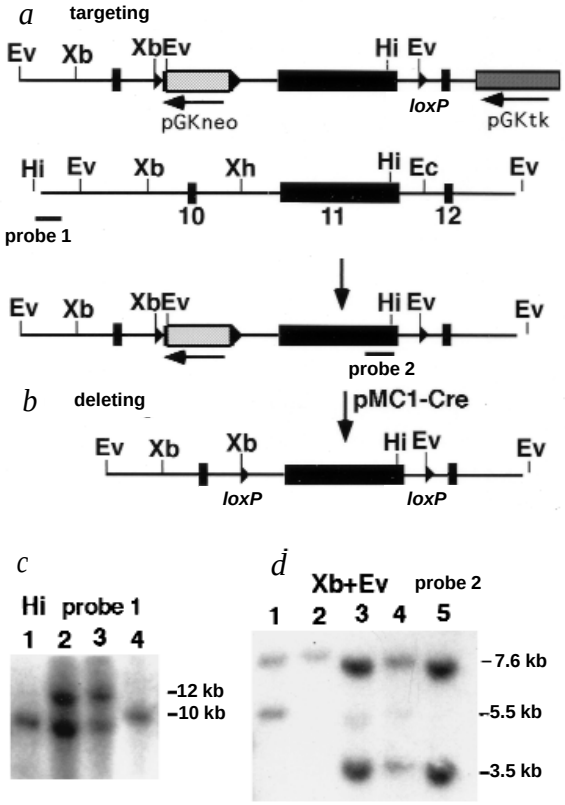

Fig. 1 Introduction of *loxP* sites into *Brca1*. ***a***, Targeting construct pLoxneo-Brca1, which contains a pLoxpneo in intron 10 and a third *loxP* site in intron 11, was used to target the *Brca1* locus. ***b***, Because the presence of the *neo* gene in intron 10 resulted in recessive embryonic lethality, the *neo* gene was removed in embryonic stem (ES) cells by transient expression of pMC1-Cre. ***c***, Southern-blot analysis to show targeted ES clones (lanes 2 and 3), which exhibited band shifts of 10–12 kb on *Hin*dIII digestion followed by hybridization with a flanking probe (probe 1). ***d***, Southern-blot analysis of DNAs from ES clones after pMC1-Cre transfection. DNAs were digested with *Xba*I plus *Eco*RV and hybridized with a 1.5-kb *Kpn*I-*Hin*dIII fragment (probe 2) that is specific to exon 11 of *Brca1*. Of 44 G418-sensitive clones examined by Southern blot, 2 clones contained recombination that just removed *neo* (lane 5) and 3 clones contained a weak band from unrecombined DNA (lanes 3 and 4). Lane 1 was DNA isolated from a clone that was heterozygous for the mutant allele. The remaining 39 clones contained recombination that deleted *neo* and exon 11 of *Brca1*. An example of these clones, which only contained a wild-type allele, is shown in lane 2. We have also removed *neo* from the mouse genome using *Ella* (adenovirus type 2 early promoter) -*Cre* transgenic mice[43], or by injecting Cre plasmid into oocytes that carry *neo*. Both methods selectively excised *neo* efficiently (data not shown).

stages of development, including pregnancy (P; days 11.5 and 16.5), lactation (L; days 1 and 10) and involution (day 10), revealed extensive Cre-mediated recombination, which peaked at approximately 50% at P16.5 (Fig. 2*c*). We detected less than 10% recombination at P11.5 and approximately 30% during lactation (data not shown). As mammary tissue consists of not only secretory epithelial cells but also fat and stromal cells, where the *Wap-Cre* transgene is inactive, the amount of epithelium that undergoes recombination may be much higher. Indeed, northern-blot analysis demonstrated that the *Brca1* transcripts were reduced in mammary tissue from P11.5 and P16.5 $Brca1^{Ko/Co}$*Wap-Cre* mice (Fig. 2*d*). Compared with the intensities of *Brca1* and *Gapd* transcripts, the amount of transcripts from $Brca1^{Ko/Co}$*Wap-Cre* glands was less than 10% of control levels.

Developmental abnormalities and increased apoptosis in *Brca1* conditional mutant glands

Development of the mammary gland proceeds in distinct stages. Ductal elongation and branching occurs mainly during puberty, whereas alveolar proliferation and differentiation take place during pregnancy[26]. Deletion of *Brca1* from mammary epithelial cells using the *Wap-Cre* transgene resulted in aberrant mammary development (Fig. 3). We assessed ductal and alveolar develop-

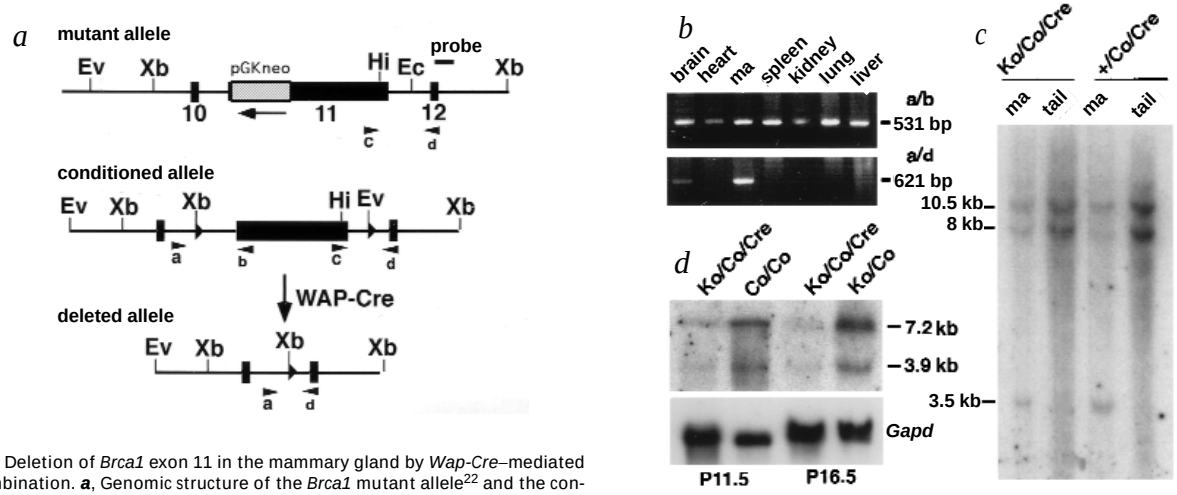

Fig. 2 Deletion of *Brca1* exon 11 in the mammary gland by *Wap-Cre*–mediated recombination. ***a***, Genomic structure of the *Brca1* mutant allele[22] and the conditional allele (Co). Arrows show positions and directions of PCR primers used for detecting products of recombination . ***b***, Cre-mediated recombination was detected predominantly in mammary gland (ma) from a P16.5 $Brca1^{Ko/Co}$*Wap-Cre* mouse. Recombination products with a much weaker intensity were also detected in brain. Recombination products, however, were only detected in mammary gland when the samples were analysed by Southern blot (data not shown). ***c***, Southern-blot analysis showing recombination of DNA from P16.5 $Brca1^{Ko/Co}$*Wap-Cre* and $Brca1^{+/Co}$*Wap-Cre* mammary glands. Both wild-type and mutant alleles were 10.5 kb, the recombined allele was 3.5 kb and the unrecombined conditional allele was 8 kb. The intensity of the 8- and 3.5-kb fragments was comparable, suggesting that approximately 50% of DNA was recombined. Because mammary tissue also contains fat and stromal cells, the remainder of the 8-kb fragment is probably derived largely from these cells. DNA was digested with *Xba*I and the probe used was a 0.7-kb *Xho*I-*Eco*RI fragment. ***d***, Northern-blot analysis showing reduced amounts of *Brca1* transcripts in mammary glands isolated from P11.5 and P16.5 $Brca1^{Ko/Co}$*Wap-Cre* mice. A cDNA probe containing exons 10 and 12 of *Brca1* was used for hybridization. Two fragments of approximately 7.2 and 3.9 kb were detected in all samples. We have previously shown that the 3.9-kb transcript is a natural Δ11 product, which creates an in-frame fusion between exons 10 and 12, whereas the 7.2-kb band may represent the full-length transcript[29]. Both bands were weaker in samples with *Wap-Cre* transgene. The same filter was also incubated with a *Gapd* probe as control.

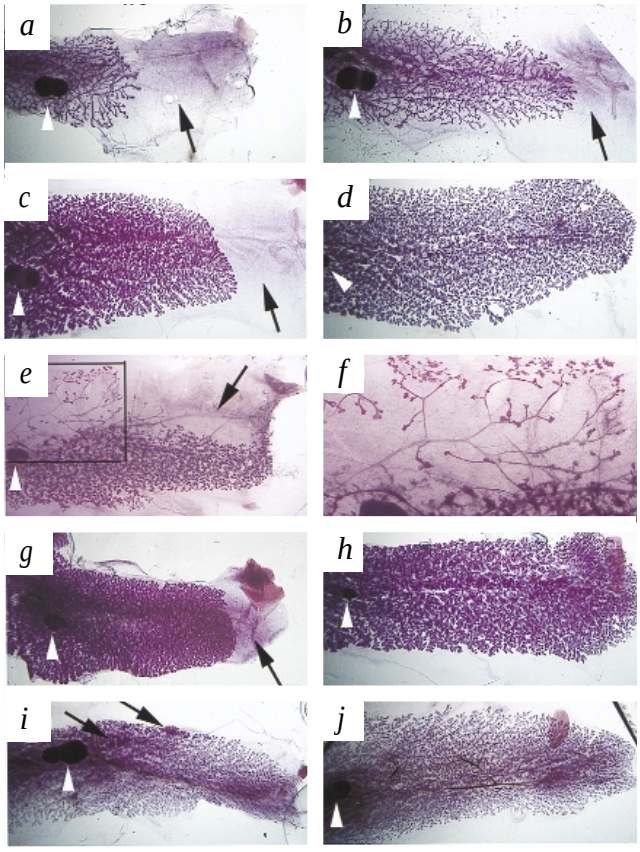

Fig. 3 Abnormal mammary gland development in *Brca1*$^{Ko/Co}$*Wap-Cre* (*a*,*c*,*e*,*f*,*g*,*i*) and control (*b*,*d*,*h*,*j*) mice. ***a***,***b***, P8.5 glands. ***c***,***d***, P16.5 glands. ***e***, A P16.5 gland showing severely underdeveloped areas. Enlarged image of area in rectangle is shown in (***f***). ***g***,***h***, L1 glands. Arrows point to empty space in fat pad. ***i***,***j***, Involution day 10 glands. Arrows indicate uninvoluted or hyperplastic areas. Lymph nodes are indicated by white arrowheads.

ment of the fourth glands at different stages of pregnancy using whole-mount preparations. At P8.5, mammary glands from *Brca1*$^{Ko/Co}$*Wap-Cre* mice were smaller than those of controls (*Brca1*$^{Ko/+}$*Wap-Cre*, *Brca1*$^{Ko/+}$ or wild type; Fig. 3*a*,*b*). At P16.5 the ductal tree in control mice completely filled the fat pad; we observed substantial alveolar development (Fig. 3*d*). In contrast, the mammary-specific mutant showed incomplete development (Fig. 3*c*). *Brca1*$^{Ko/Co}$*Wap-Cre* mice (n=4) that exhibited more than 30% Cre-mediated recombination had fat pads that were less than 80% filled. Despite continued hormonal signalling throughout pregnancy, mutant mammary tissue failed to penetrate further into the fat pad. In one extreme case found at P16.5, *Brca1*$^{Ko/Co}$*Wap-Cre* mammary tissue failed to develop in one-half of the fat pad (Fig. 3*e*,*f*). Abnormal ductal morphogenesis was also observed in mutant mammary glands at other stages of development, including lactation (Fig. 3*g*,*h*) and involution (Fig. 3*i*,*j*). Residual alveolar structures sometimes remained in involuting *Brca1*$^{Ko/Co}$*Wap-Cre* mammary glands (Fig. 3*i*), but not in control glands (Fig. 3*j*). Mammary glands of *Brca1*$^{Ko/Co}$*MMTV-Cre* females from different developmental stages also showed similar defects (Fig. 4*a*–*d*). Despite these abnormalities, dams were able to nurse their litters.

To identify the mechanisms underlying the restricted development of the mammary gland, we performed TUNEL assay to determine the apoptotic status of mammary tissue within four hours after parturition. We observed very low levels of apoptosis in 9 control glands (Fig. 4*e*), whereas extensive apoptosis occurred in 3 of 6 *Brca1*$^{Ko/Co}$*MMTV-Cre* (Fig. 4*f*) and 1 of 3 *Brca1*$^{Ko/Co}$*Wap-Cre* (data not shown) glands. Northern-blot analysis indicated that the extent of cell death correlates with amount of recombination. The 4 glands that exhibited extensive apoptosis had *Brca1* transcripts that were reduced 60–80%, and 2 other glands that demonstrated apoptosis to a lesser extent had *Brca1* transcripts that were reduced by approximately 30% (data not shown).

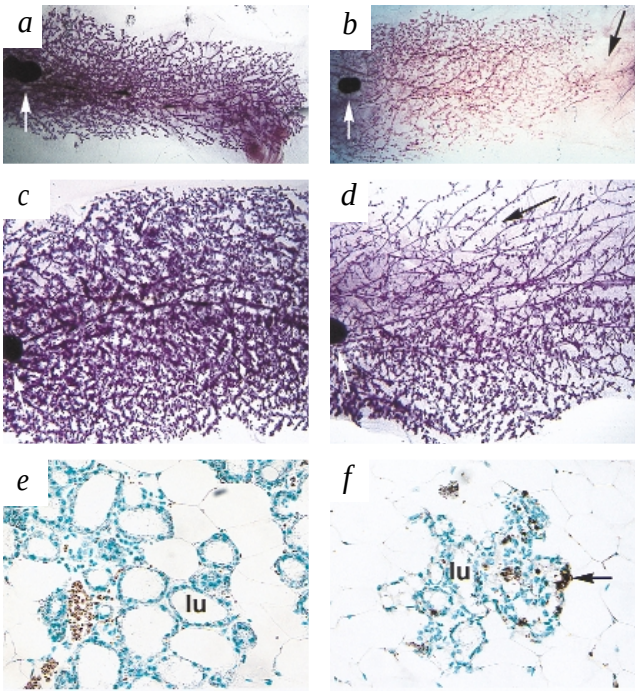

Fig. 4 Abnormal mammary gland development in *Brca1*$^{Ko/Co}$*MMTV-Cre* mice. P11.5 wild-type (***a***) and conditional mutant (***b***) mammary glands are shown. A black arrow points to unfilled fat pad. P16.5 wild-type (***c***) and conditional mutant (***d***) mammary glands are shown. A black arrow indicates areas where the ductal branches are sparse. White arrows in (*a*–*d*) indicate lymph nodes. *e*,*f*, L1 wild-type (***e***) and conditional mutant (***f***) glands. TUNEL assay for apoptosis revealed that approximately 20% of cells in *Brca1*$^{Ko/Co}$*MMTV-Cre* glands (*f*) and none in control (*e*) glands undergo apoptosis (*f*, arrow). lu, lumen.

Mammary-gland tumour formation in *Brca1* conditional mutant glands

Germline mutations in *BRCA1* are responsible for approximately 50% of human hereditary breast cancers[4]. *Brca1*$^{Ko/Co}$*Wap-Cre* and *Brca1*$^{Ko/Co}$*MMTV-Cre* females were therefore continuously mated and monitored for the formation of mammary-gland tumours. Whole-mount staining and/or histological examination of the fourth glands isolated from 20 female mice of 2–10 months revealed no tumour formation. Hyperplastic areas, however, were found in four conditional mutant mammary glands, but not in control glands (Fig. 5*a*,*c*). Further analysis of mice at later ages (10–13 months) revealed 3 of 10 *MMTV-Cre* and 2 of 13 *Wap-Cre* females developed mammary tumours of diverse types (Fig. 5*e*,*f*).

Mammary-gland tumour formation is accompanied by rearrangements of chromosome 11

The low frequency and long latency of mammary tumour formation led us to determine whether the tumours were a consequence of Cre-mediated deletion. Using primers that

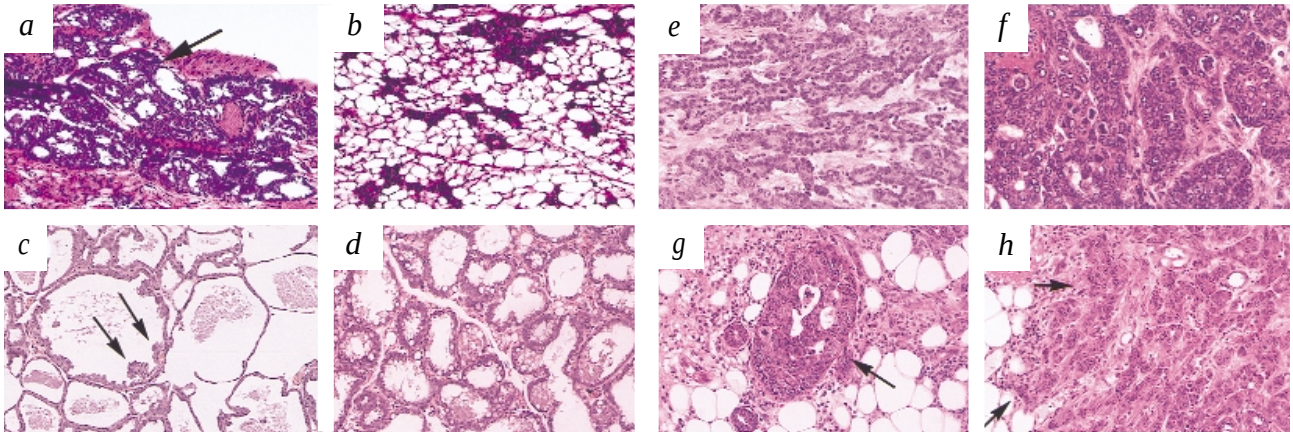

Fig. 5 Tissue-specific mutant of *Brca1* results in mammary-gland tumour formation. Histological sections of alveolar hyperplasia (arrow) from an involuting day-10 gland of a $Brca1^{Ko/Co}$*Wap-Cre* female mouse (***a***) and an involuting day-10 wild-type gland (***b***). Histology of a papillary hyperplasia (arrows) from a lactating $Brca1^{Ko/Co}$*MMTV-Cre* female mouse (***c***) and a control lactating gland (***d***). Histology showing a tubular adenocarcinoma from a 10-month-old $Brca1^{Ko/Co}$*MMTV-Cre* female (***e***) and a solid adenocarcinoma from a 13-month-old $Brca1^{Ko/Co}$*Wap-Cre* female. A ductal carcinoma *in situ* (***g***, arrow) and a highly invasive adenocarcinoma (***h***, arrows point to the border between the tumour and the surrounding mammary tissues) from a 7-month-old $Brca1^{Ko/Co}$*MMTV-CreTrp53*$^{+/-}$ female mouse.

simultaneously amplify both the mutant allele (794 bp) and the unrecombined conditional allele (854 bp, due to the presence of the 60-bp *loxP* site), we found that the intensities of unrecombined conditional alleles were reduced in 3 tumours (Fig. 6*a*, primers c/d, arrows), suggesting that most cells had undergone Cre-mediated recombination. We were unable to detect the expected PCR product of the recombined conditional allele in two of these samples, whereas such product was detected in normal glands from the same animals (Fig. 6*a*, primers a/d). Although this observation suggests the loss of Cre-mediated recombination product in these tumours, we detected the loss of a $Brca1^{Ko}$ allele in another tumour (Fig. 6*a*, double arrow).

Our observations suggest that in addition to the Cre-mediated deletion of *Brca1* exon 11, further changes are also required for tumour formation. This may include the rearrangement or loss of *Brca1* and surrounding regions of chromosome 11, which harbours several other known tumour-suppressor genes, including *Trp53* (ref. 27). To determine if chromosomal changes are present in these tumour cells, we performed FISH on metaphase spreads using a whole chromosome-11 painting probe. A representative cell from the tumour is shown (Fig. 6*c*). Most cells were near triploid, and contained 2 copies of chromosome 11, and 2 different translocated chromosomes, 1 involving the distal and 1 the proximal portion of chromosome 11. Two copies of each translocation were present, and the breakpoints in chromosome 11 were located in bands B–D, which contain both *Brca1* (11D; ref. 28) and *Trp53* (11B; ref. 27). To determine whether *Trp53* is indeed involved, we studied its expression in tumours and found it to be altered in 2 of 3 tumours. The normal transcript of *Trp53* is approximately 2 kb, but we detected a message of approximately 4 kb in 1 tumour and no transcript in the other (Fig. 6*b*). These data suggests a possible role for *Trp53* in Brca1-associated tumorigenesis, which may include the loss or rearrangement of the gene. Rearrangements on other chromosomes such as deletions, dicentric chromosomes and chromosome breakage are also present in tumour cells (data not shown), indicating that the genomic instability we observed in *Brca1*-Δ11 deficient embryos[29] is recapitulated in these tumours.

A *Trp53*-null allele accelerates mammary-gland tumour formation in *Brca1* conditional mutant glands

Alterations of *Trp53* expression in *Brca1* conditional mammary gland tumours are reminiscent of human BRCA1 familial breast tumours, which frequently contain *TP53* mutations[30,31]. Thus, it is important to address whether *TP53* alterations are relevant to breast tumour progression. *TP53* is a well-known tumour-suppressor gene that is mutated in approximately 50% of all human tumours[32]. It may be that *TP53* alterations represent one of the genetic changes required for BRCA1-associated tumorigenesis. To test this hypothesis, we introduced a loss-of-function allele of *Trp53* (ref. 33) into *Brca1* conditional mutant mice. Of 11 $Brca1^{Ko/Co}$*MMTV-CreTrp53*$^{+/-}$ females examined between 6 and 8 months of age, 8 developed mammary gland tumours with several distinct histopathologies (Fig. 5*g*,*h*, and data not shown). Two mice had more than one tumour (two and three tumours, respectively), and the remaining six carried only one tumour. No tumours were observed in over 30 age-matched control mice (*Trp53*$^{+/-}$, $Brca1^{Ko/+}$*MMTV-CreTrp53*$^{+/-}$, $Brca1^{+/Co}$*MMTV-CreTrp53*$^{+/-}$, or $Brca1^{Ko/Co}$*Trp53*$^{+/-}$) that had gone through at least 3 pregnancies. Southern-blot analysis of five tumours indicated that all had undergone Cre-mediated recombination (Fig. 6*e*). Our data also showed that 4 of 5 tumours lost or partially lost the wild-type *Trp53* allele and 2 tumours lost either the $Brca1^{Ko}$ allele or the product generated by Cre-mediated recombination (Fig. 6*d*,*e*). Compared with the tumour profile shown earlier, these data demonstrated that the loss of *Trp53* accelerated tumour formation in mammary glands from mice with a specific deletion of *Brca1*.

Discussion

We have assessed the function of Brca1 in mammary-gland development and neoplasia using the Cre-*loxP* recombination system under control of the *Wap* and *MMTV* promoters. In the absence of the full-length Brca1 isoform, mammary tissue fails to fully develop during pregnancy. The ductal tree is incompletely formed and does not fully penetrate the mammary fat pad. Cre-mediated excision of *Brca1* exon 11 is frequently accompanied by increased apoptosis. Notably, mammary-gland tumours develops in $Brca1^{Ko/Co}$*Wap-Cre* and $Brca1^{Ko/Co}$*MMTV-Cre* mice after a long latency. As the levels of *Brca1* transcripts were reduced to as

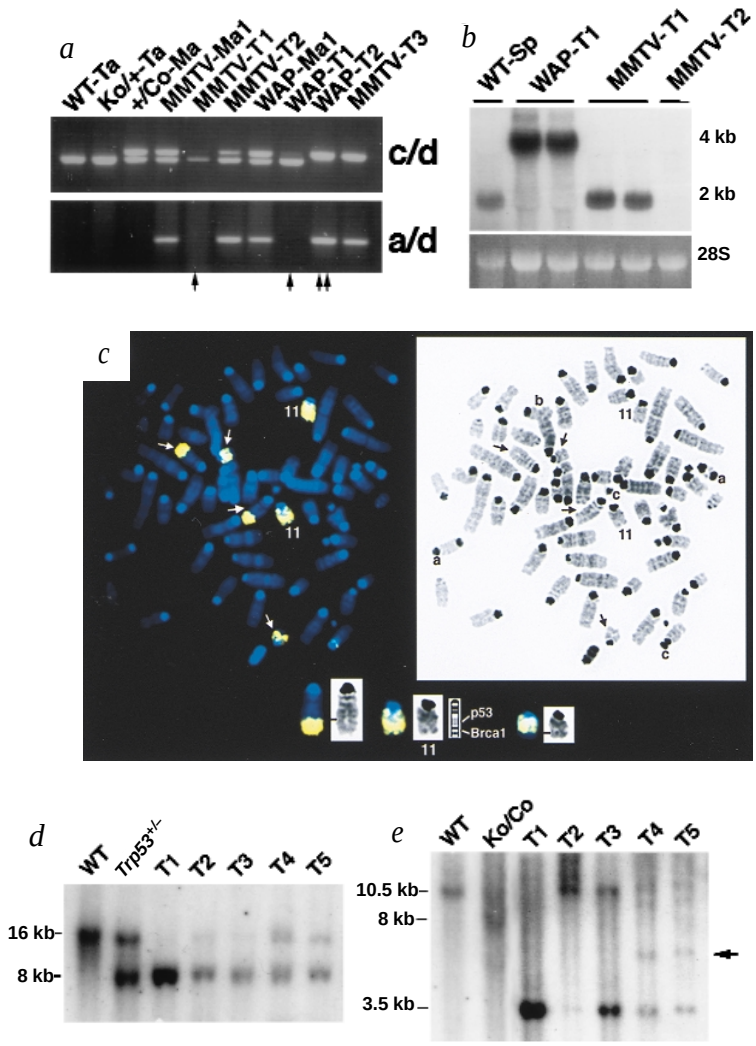

Fig. 6 Genetic instability in tumours. ***a***, PCR analysis of tail (Ta), normal mammary glands (Ma) and tumours (T). *Brca1*$^{Ko/Co}$*MMTV-Cre* tumour 1 (MMTV-T1, arrow) and *Brca1*$^{Ko/Co}$*Wap-Cre* tumour 1 (WAP-T1, arrow) showed reduced intensities of the unrecombined conditional allele (top; primers c/d). The predicted Cre-mediated recombination product, however, is not observed in these tumours (primers a/d). One *Brca1*$^{Ko/Co}$*Wap-Cre*-tumour (WAP-T2, double arrow) did not show PCR product from the *Brca1*^{Ko} allele, suggesting that the allele may be lost. The last lane (MMTV-T3) shows a tumour from a *Brca1*$^{Co/Co}$*MMTV-Cre* female that does not carry the *Brca1*^{Ko} allele as predicted. ***b***, Northern-blot analysis using the entire mouse *Trp53* cDNA as a probe[44]. *Trp53* transcripts were either increased in size from 2 kb to 4 kb (WAP-T), lost (MMTV-T1) or unchanged (MMTV-T2). For WAP tumour-1 and MMTV tumour-1, 2 samples with RNAs prepared from 2 different parts of the tumours are shown. The first lane is RNA isolated from a wild-type spleen (WT-Sp) as control. ***c***, Metaphase spread from a MMTV tumour labelled with a chromosome-11 painting probe (yellow) and counterstained with DAPI (blue). Enlarged copies of the translocated chromosomes are shown at the bottom, flanking a copy of the 'unrearranged' chromosome 11. Approximate breakpoints are indicated by black bars and the locations of *Trp53* (p53) and *Brca1* are indicated on the ideogram. A Giemsa-stained image of the same metaphase spread is shown on the right. Chromosome paints were prepared as described[22]. FISH analysis on all 3 tumours presented in (*b*) was performed and chromosome-11 rearrangements were observed in all cases. ***d***, Loss of wild-type *Trp53* (16-kb fragment) in 4 of 5 tumours examined by *Eco*RI digestion. ***e***, Loss of *Brca1*^{Ko} allele in tumour 1 (T1; 10.5 kb) and Cre-mediated recombination product (T2, 3.5 kb) in *Brca1*$^{ko/Co}$*MMTV-CreTrp53*$^{+/-}$ mammary gland tumours. (Both WT and *Brca1*^{Ko} alleles are 10.5 kb, *Brca1*^{Co} allele is 8 kb and recombination product is 3.5 kb upon *Xba*I digestion as shown in Fig. 2.) T4 and T5 contain an unidentified fragment (arrow).

low as 10% of controls, and Brca1 is a nuclear protein[14] that may act in a cell-autonomous fashion, it is unlikely that the relatively low incidence of tumour formation is due to the presence of residual Brca1. Rather, it indicates that the loss of full-length Brca1 does not result in a mutator phenotype observed in tumours with microsatellite instability[34]. We have previously shown that alternative splicing at exon 11 of *Brca1* generates two major isoforms, that is, the full-length isoform and a Δ11 isoform. Our conditional allele removes the full-length transcript, but the Δ11 form is still transcribed. Using an isoform-knockout approach, we have recently demonstrated that the full-length isoform of BRCA1 has an essential role in maintaining genetic stability[29]. Mouse embryonic fibroblast cells homozygous for the *Brca1* exon-11 deletion exhibited cellular proliferation defects and genetic instability caused by a defective G_2-M cell-cycle checkpoint and abnormal centrosome duplication[29]. Thus, *Brca1* is likely to act as a 'caretaker' whose inactivation does not directly promote tumour initiation. Instead, it increases mutation rates of all genes, including tumour-suppressor genes and oncogenes[35].

We propose the following model for the role of Brca1 in mouse mammary-gland tumorigenesis (Fig. 7). Assuming that Brca1 functions similarly in embryonic and mammary gland development, the disruption of *Brca1* in the mammary gland should inhibit its growth. The observations that mammary glands carrying the conditional mutant exhibited defective developmental patterns and increased apoptosis are consistent with this notion. Loss of the BRCA1 full-length isoform, however, must at the same time trigger genetic instability and allow chromosome alterations to occur. If accumulating changes result in events that overcome proliferation defects and apoptosis, which may be caused by the activation of p53, these cells may now have a growth advantage resulting in hyperplasia, as observed in these mice. Subsequent genetic instability may result in changes that eventually lead to tumorigenesis. Because Brca1-related tumorigenesis is characterized by an initial growth disadvantage, followed by subsequent genetic alterations, it is not unexpected that tumorigenesis occurs after a long latency.

Our data have linked the *Trp53* tumour-suppressor gene to Brca1-associated tumorigenesis. Using chromosomal karyotyping and FISH analysis, we demonstrated that chromosome 11, which harbours *Trp53*, is a target for genetic alterations. We then showed that *Trp53* mRNA is either deleted or altered in 2 of 3 tumours analysed. Our observations suggest that *Trp53* may be one of the genetic factors that inhibit tumour progression in *Brca1* conditional mutant mammary glands. We directly tested this hypothesis by introducing one *Trp53*-null allele[33] into conditional mutant mice. The acceleration in tumour formation observed in *Brca1*$^{Ko/Co}$*MMTV-CreTrp53*$^{+/-}$ female mice provides direct genetic evidence that p53 is involved in Brca1-asso-

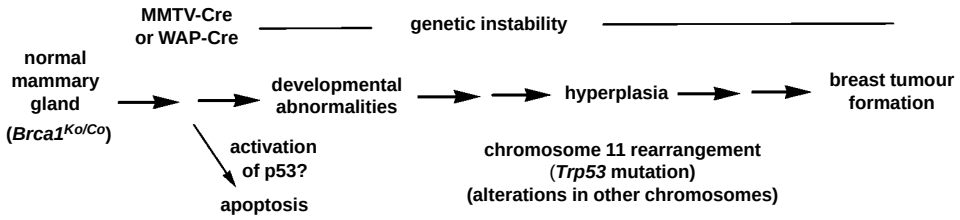

Fig. 7 Tumorigenesis in mammary-gland–specific disruption of *Brca1*. The prominent features of Cre-mediated deletions of *Brca1* exon 11 are developmental abnormalities and increased apoptosis, which may be a consequence of p53 activation. The loss of Brca1 also results in genetic instability that allows further genetic alterations to occur. Our data show that *Trp53* is a major genetic factor involved in Brca1-associated tumorigenesis. Thus the genetic instability due to the loss of Brca1 inactivates p53, which in turn accelerates tumour formation. Many unidentified factors may also be involved in Brca1-related tumourigenesis. Double arrows represent multiple events needed for hyperplasia and tumour formation.

ciated tumorigenesis. It was recently shown that mice heterozygous for both *Brca1* and *Trp53* mutations ($Brca1^{+/-}Trp53^{+/-}$) developed mammary gland tumours at a low frequency on γ-irradiation[36]. This is consistent with our observation that the loss of p53 accelerates Brca1-associated tumorigenesis. Notably, human BRCA1 familial breast tumours frequently exhibit *TP53* mutations[30,31]. If our model for mouse Brca1-related tumour progression is applicable to the human situation, we suggest that the genetic instability associated with the loss of BRCA1 triggers mutation of *TP53*, which in turn overcomes cellular proliferation defects and facilitates tumour formation.

Identification of *Trp53* as a major genetic factor in Brca1-associated tumorigenesis does not necessarily exclude involvement of other factors. Indeed, high degrees of aneuploidy and multiple structural aberrations were also detected on other chromosomes other than chromosome 17 in human[37] and chromosome 11 in mouse (unpublished data). Mice with deletion of *Brca1* in a tissue- and time-dependent manner provide a tool to identify these additional factors important for the initiation and progression of breast cancer. They should also serve as a model to study genetic pathways dysregulated in human hereditary breast cancer, and facilitate therapeutical and chemoprevention studies.

Methods

Targeting vector. Recombinant phage containing overlapping genomic DNA of the *Brca1* locus were isolated from a 129SVJ-mouse library (Stratagene). To construct the targeting vector for *Brca1*, a 3.5-kb *Eco*RV-*Xho*I fragment 5′ to exon 11 of *Brca1* was subcloned into the *Xba*I and *Eco*RI sites of pLoxpneo (ref. 38). The resulting construct was cleaved with *Xho*I and *Not*I, followed by insertion of a 5.5-kb *Xho*I-*Not*I fragment (the *Not*I site is from the polylinker of the phage vector). A single *loxP* site was inserted into the *Xho*I-*Not*I fragment at its *Eco*RI site located in intron 11. The finished targeting construct is designated pLoxpneoBrca1.

ES cell manipulation. TC1 ES cells[39] were transfected with *Not*I-digested pLoxpneoBrca1 and selected with G418 and FIAU as described[40]. We removed ploxpneo from targeted ES cells as described[41], with the following modification to enrich Cre-mediated recombination. After electroporation using 5 μg of DNA per 10^7 cells, we plated ES cells heterozygous for the pLoxpneoBrca1-targeted allele at densities of 500–3,000 cells/10-cm plate. After 6 d, 384 clones were picked and duplicated in 96-well plates. G418 was added to one set of plates. After 3 d, we amplified G418-sensitive clones (44/384) from the second set of the plates for further analysis. Two clones were found that contained recombination that removed only *neo*. Three additional clones contained a weak band resulting from unrecombined DNA. All others (39/44) contained recombination that deleted *neo* and *Brca1* exon 11.

Mating and genotyping mice. Chimaeric mice were mated with NIH Black Swiss females (Taconic) to screen for germline transformation. Mice carrying a *Brca1* conditional allele were genotyped by Southern analysis or PCR (60 ºC, 30 s; 72 ºC, 1 min; 94 ºC, 30 s; 30 cycles). For PCR analysis, the following primers were used: (primer a, 5′–CTGGGTAGTTTGTAAGCATCC–3′; primer b, 5′–CAATAAACTGCTGGTCTCAGGC–3′; primer c, 5′–GGAAATGGCAACTTGCCTAG–3′; and primer d, 5′–CTGCGAGCAGTC TTCAGAAAG–3′). Primers 'a' and 'b' flank the *loxP* insertion site in intron 10, and amplify a 470-bp fragment from wild-type *Brca1* and 530 bp from the conditional allele. Mice carrying the *Brca1*-null allele[22], *Trp53* (ref. 33) and *Wap-Cre* or *MMTV-Cre* (ref. 25) were genotyped as described. $Brca1^{Ko/Co}$*Wap-Cre*, $Brca1^{Ko/Co}$*MMTV-Cre* or $Brca1^{Ko/Co}$*MMTV-Cre*$Trp53^{+/-}$ females were kept with males for continuous mating. Their pregnancy times were recorded. When sacrificed, one of the fourth glands was used for whole-mount preparation and the other for DNA, RNA and/or histology analysis.

Northern blots, TUNEL assay and whole-mount staining of mammary glands. RNA was isolated from mammary glands of P11.5–L1 females using RNA Tet-60 based on the protocol recommended by the manufacturer (Tel-Test "B"). We prepared poly(A)$^+$ RNA using a kit (Pharmacia). Poly(A)$^+$ RNA (~10 μg) from each sample was electrophoresed on a 1% agarose gel and transferred to a Gene-Screen filter. TUNEL assay was carried as recommended by the manufacturer (Oncor). We carried out whole-mount staining of mammary glands as described[42].

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